Cancer Management and Research

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Cadherin-11 is inactivated due to promoter methylation and functions in colorectal cancer as a tumour suppressor

This article was published in the following Dove Press journal:
Cancer Management and Research

Shiyun Yuan[1]
Lin Li[1]
Shili Xiang[2]
Hexun Jia[3]
Tao Luo[1]

[1]Department of Geriatrics, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China; [2]Department of Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China; [3]Office of academic, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China

Background: The *cadherin-11* (*CDH11*, OB-cadherin) gene is a member of the cadherin family and is located on chromosome 16q22.1. Previous studies have revealed that cadherins play significant roles in the development of many human malignancies. Increasing evidence has identified *CDH11* as a functional tumour suppressor, which is commonly silenced by promoter methylation, but the functions of this gene in colorectal cancer (CRC) have been unclear.

Methods: The *CDH11* expression in primary CRC tissues and cell lines was investigated by qRT-PCR, RT-PCR and immunohistochemistry. The promoter methylation status of *CDH11* was measured by methylation-specific PCR (MSP). Cell proliferation assay, colony formation assay, flow cytometry analysis, wound-healing assay, transwell assay and in vivo experiments were used to investigate the function of *CDH11* in CRC. The mechanisms of *CDH11* also were explored by western blots.

Results: Our study suggests that *CDH11* downregulation in CRC due to its promoter methylation and induced cell cycle arrest in G0/G1 phase and apoptosis, suppressing tumor cell proliferation, colony formation, migration and invasion by affecting the *NF-kB* signaling pathway.

Conclusion: Overall, *CDH11* may be considered as a functional tumour suppressor gene (TSG) in CRC, *CDH11* has the potential to serve as a valuable prognostic marker for colorectal cancer.

Keywords: cadherin 11, colorectal cancer, methylation, NF-kB, tumour suppressor

Introduction

Colon and rectum cancer (colorectal cancer, CRC) is the third most-common cancer, and CRC accounting for 6.1% (1,800,977) of all cancer cases and 9.2% (881,000) of cancer deaths in 2018, according to the latest global cancer statistics from the International Agency for Research on Cancer.[1] Mounting more evidence has suggested that screening and early diagnosis is associated with a reduction in CRC. However, the relevant methodologies are still limited, underlining the importance of finding a reliable biomarker for an early detection of this disease.[2] Six classical cadherin family genes (six cadherin clusters) are located on 16q22.1-16q24.3 and comprise of six cell-cell adhesion molecules mediating intercellular adhesion by Ca^{2+}-dependent homophilic interactions. The family members include *CDH1(E-cadherin)*, *CDH3(P-cadherin*), *CDH5*(*VE-cadherin*), *CDH8, CDH11*(*OB-cadherin)* and *CDH13*(*H-cadherin*).[3] *CDH1* and *CDH13* have been identified as functional tumour suppressors, which inhibit cell proliferation and invasiveness in most cancer cell lines and reducing tumour growth in in vivo models.[4,5]

Correspondence: Tao Luo
Department of Geriatrics, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China
Tel +861 365 059 1212
Email loto1314@yeah.net

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/CMAR.S193921

The specific mechanism of CRC tumourigensis involves the activation of an oncogene and the inactivation of a tumour suppressor gene. The tumour suppressor gene inactivation is closely related to epigenetic changes.[6] A previous study has identified DNA copy number aberrations and an ~1 Mb hemizygous deletion at 16q21-22.1 in tumour cell lines by performing 1-Mb array comparative genomic hybridization (aCGH), indicating that *CDH11*, the only known gene located at this deletion, could be 16q21-22.1 deletion-related candidate TSG.[3] Researchers have found that *CDH11* is downregulated and frequently methylated in multiple types of tumours.[7–10] However the roles of *CDH11* in CRC have not been elucidated. Therefore, in this study, we investigated the *CDH11* expression, promoter methylation status, biological functions and related molecular mechanisms in CRC.

Material and methods

Cells lines and tumour samples

Six human colorectal cancer cell lines (ie, LoVo, HCT116, SW480, HT-29, CaCo-2, RKO) were used. The CRC cell lines HT-29 and HCT116 were provided by Professor Q.Tao at the Chinese University of Hong Kong, and the SW480, LoVo, CaCo-2, RKO cell lines were purchased from the Chinese Academy of Sciences. And the use of the gifted cell lines was approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University. The cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Karlsruhe, Germany) with 10% fetal bovine serum (FBS) (ExCell Bio, Shanghai, China), and maintained at a humidified atmosphere of 5% CO_2 at 37 °C.[11] The CRC tissues and their corresponding adjacent tissues were collected from surgical patients at the First Affiliated Hospital of Chongqing Medical University (Chongqing,China), and all tissues were diagnosed and verified at the Pathology Department, Chongqing Medical University (Chongqing, China). The samples were immediately snap-frozen in liquid nitrogen and then stored at −80 °C until analysis. The study protocol was authorized under the guidelines of the ethics committee of the First Affiliated Hospital of Chongqing Medical University. This study was conducted in accordance with the Declaration of Helsinki and all patients signed an informed consent form.

Nucleic acid and protein extraction

Total RNA was extracted from tissues and cell lines using TRIzol® reagent (LifeTechnologies, Carlsbad, CA, USA). Genomic DNA was isolated from tissues and cell lines using the QIAamp DNAMini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA and RNA were stored at −80 °C after measuring their concentrations of them using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). The experimental and control groups of the HCT116 and LoVo cells were lysed using a protein extraction reagent (Thermo Scientific) that contained the protease inhibitors, phenylmethane sulfonyl fluoride, and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and the lysate was then homogenized using an Ultrasonic Cell Grinder (Scientz, Ningbo). The supernatant was collected after centrifugation, and protein concentration of protein in the supernatant was determined using the BCA protein kit (Thermo Scientific).[12]

Semiquantitative polymerase chain reaction (PCR) and quantitative RT-PCR (qRT-PCR)

Semiquantitative PCR and quantitative PCR were used to determine expression of *CDH11* in CRC cells and tissues. The RNA (1 μg) was reverse transcribed to 20 μg of cDNA using the Reverse Transcription system (Promega, Madison, WI, USA). For semiquantitative PCR, the *CDH11* gene was amplified using GoTaq DNA polymerase (Promega). The protocol included an initial denaturation at 95 °C for 2 min, followed by 34 amplification reaction cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s), with a final extension at 72 °C for 3 min, and used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control. The primer sequences are listed in Table 1. Quantitative PCR was performed with an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Hong Kong, China) with *GAPDH* as a control.[11] Each sample was tested in triplicate.

Bisulfite treatment and methylation-specific PCR (MSP) analyses of CDH11

Bisulfite modification of DNA and methylation-specific PCR (MSP) were carried out in accordance with previously described methods.[13] The primers used for MSP are listed in Table 1. MSP for the *CDH11* promoter was performed using AmpliTaq®-Gold DNA Polymerase (Applied Biosystems) mixed with bisulfite-treated DNA, $MgCl_2$ and deoxynucleotide triphosphates for the MSP amplification reaction. The

Table 1 The primers used in this study

PCR	Primer	Sequences (5′-3′)	Product size (bp)	Annealing temperature(°C)	Cycles
RT-PCR	CDH11F	tggcagcaagtatccaatgg	200bp	55	33
	CDH11R	tttggttacgtggtaggcac			
MSP	CDH11m3	ttatttttgttattagcgcgttc	123bp	60	40
	CDH11m4	ccattcacaaatcaacgacg			
	CDH11u3	tttttatttttgttattagtgtgttt	128bp	58	40
	CDH11u4	tcccattcacaaatcaacaaca			

Abbreviations: PCR, semiquantitative polymerase chain reaction; qRT-PCR, quantitative polymerase chain reaction; MSP, methylation-specific PCR.

reaction conditions were as follows: initial denaturation for 10 min at 95 °C, then 40 cycles consisting of 95 °C for 30 s, 60 °C (methylated reactions) or 58 °C (unmethylated reactions) for 30 s, 72 °C for 30 s, and a final extension for 5 min at 72 °C. The final products were identified on a 2% agarose gel containing 100 bp DNA markers (MBI Fermentas Vilnius, Lithuania), and then recorded using a Molecular Imager (Bio-Rad, Hercules, CA, USA).[11,12]

5-Aza-2′-deoxycytidine (Aza) and trichostatin a (TSA) treatment

As previously reported,[12,14] the cells were treated with a DNA methyltransferase (DNMT) inhibitor,5-Aza-2′-deoxycytidine (Aza) (Sigma-Aldrich, Steinheim, Germany), in the dark at a final concentration of 10 mmol/l for 3 days, and further treated with 100 nmol/l trichostatin A(TSA) (Cayman Chemical, Ann Arbor, MI, USA), a histone deacetylase inhibitor, for another 1 day. Other groups had cells treated only with Aza (Sigma-Aldrich) in the dark at a final concentration of 10 mmol/l for 3 days or TSA at a final concentration of 100 nmol/l for 1 day. Cells were then collected for RNA and DNA extraction.

Construction of CDH11-overexpressing LoVo and HCT116 cell lines

CRC cell lines were transfected with pcDNA3.1-*CDH11*or pcDNA3.1 (+) vectors using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free RPMI-1640 medium; 4–6 h later, the medium was replaced with fresh growth medium containing 10% FBS for 48 h. Then, CRC LoVo and HCT116 cells were selected in the medium containing 10% FBS with G418 (Invitrogen/Gibco) at a final concentration of 5 μl/ml or 3 μl/ml approximately 20 days later. RT-PCR and western blot analyses were confirmed stable overexpression of *CDH11*. *CDH11*-expressingplasmid was provided by Professor Qian Tao at the Chinese University of Hong Kong.

Cell proliferation assay

Cell proliferation was assessed by CCK-8 assay. LoVo and HCT116 cells stably expressing *CDH11* or pcDNA 3.1 were seeded in 96-well plates ($2x10^3$ cells/well) with 100 μl/ml of complete culture medium. Then, the medium in each well was replaced with 100 μl RPMI-1640 (10% FBS) containing 10 μl CCK-8 solution and incubated at 37 °C for 2 h in the dark. The absorbance was measured at 450 nm using a microplate reader (Multiskan MK3; ThermoFisher Scientific, former Fermentas, Schwerte, Germany) at 24, 48 and 72 h. All experiments were repeated three times.

Colony formation assay

Stably expressing cells were planted in 6-well plates at $1×10^3$ cells/well and cultured for 14 days, then fixed in 4% paraformaldehyde for 30 min, and stained with Gentian Violet (ICM Pharma, Singapore, Singapore) for 30 min. Finally, the cells were scanned using a CanoScan8800F (Canon, Tokyo, Japan). Surviving colonies (>50 cells/colony) were counted using the ImageJ (V.1.8.0) software (National Institutes of Health, Bethesda, MD, USA) for the analysis. All experiments were assessed in triplicate.

Flow cytometry analysis of cell cycle and apoptosis

Stable HCT116 and LoVo cells were collected, digested, rinsed with phosphate-buffered saline (PBS) and centrifuged at 800 rpm for 5 min, then fixed with ice-cold 70% ethanol at 4 °C overnight. Then the cells were stained with 50 mg/l propidium iodide (PI) (Beyotime) for 30 min at 4 °C in the

dark. For the cell apoptosis measurements, cells were washed, collected, resuspended in PBS, stained with Annexin V-FITC (BD Pharmingen, San, Jose, CA, USA) and PI in the dark for 15 min, and analysed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analysed using the Cell Quest software (BD Biosciences, San Jose, CA, USA).

Wound-healing assays

Stably expressing cells were planted in six-well plates until 95% confluent. Asterile P-20 pipette tip was used to scratch wounds across each well. Cells were then washed three times with 1 ml/well PBS to carefully remove cell debris and cultured in serum-free media. The cell-free wound area was photographed at 0, 24, 48 and 72 in LoVo culture, and the images of the wound closure areas of HCT116 cells were observed at 0, 24, 48 and 60 h using a light microscope (CTR4000; Leica, Germany) at 100× magnification.

Transwell assays

Transwell chambers (8 μm pore size; Corning, New York, NY, USA) with or without a Matrigel (BD Biosciences) barrier added to the top chamber were used to detect the migratory and invasive abilities of CRC cells.[15] HCT116 and LoVo cells stably expressing *CDH11* or pcDNA 3.1 were collected, washed twice in serum-free medium, then2×10^4 cells suspended in 100 μl were added into the upper transwell chamber, and 800 μl of medium with 10% FBS was added to the lower chamber. After incubation at 37 °C and 5% CO2 for 48 h, cells at the lower side of the inserts were fixed in 4% paraformaldehyde for 30 min and stained with crystal violet (DC079; Genview, Beijing, China) for 20 min at room temperature. Non-migratory cells on the upper side of the chamber were wiped off with a cotton bud. Cells from 5 random fields were counted under a microscope at 400× magnification (CTR4000; Leica, Wetzlar, Germany).

Western blot analysis

A total of 40 mg of protein lysate was subjected to western blotting as previously described.[16] The primary antibodies used were as follows: *CDH11* (1:1,000, ab151302; Abcam, Cambridge, UK), *cleaved-caspase 3* (1:500, WL03089; Wanleibio, Shenyang, China), *cleaved-caspase 9* (1:500, WL01838; Wanleibio, Shenyang, China), *cleaved-PARP* (1:1,000, 7851; Cell Signaling Technology), *BAX* (5023; Cell Signaling Technology), *NF-κB* (ab16502; Abcam, Cambridge, UK), *phospho-NF-κB* (3031s; Cell Signaling Technology), *Bcl-2* (2872T; Cell Signaling Technology), *Bcl-XL* (2762S; Cell Signaling Technology), and *β-actin* (LK-ab008-100; Liankebio, China) was used as control. Anti-mouse *IgG* (7076, 1:3,000 Cell Signaling Technology) and anti-rabbit *IgG* (7074, 1:2,000 Cell Signaling Technology) horseradish peroxidase conjugate secondary antibodies were used. Protein blots were analysed using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and the membranes were visualized with a Las-4,000 Imaging System (Medical Systems, Fujifilm Global, Tokyo, Japan).

In vivo tumourigenicity

HCT116 cells stably expressing *CDH11* or pcDNA 3.1 was (5×10^6 cells in 0.15 ml serum-free medium) were injected subcutaneously into six4-week-old nude mice (purchased from the Experimental Animal Center of Chongqing Medical University, China). The length and width were measured every 3 days using a microcaliper, and the tumour volume (mm^3) was calculated using the following equation: volume =0.5× length × $width^2$ When the tumour length reached 1.5 cm, the tumours were removed, and mice were euthanatized. All removed tumor were weighed immediately before fixing in 4% paraformaldehyde, dehydrated and embedded in paraffin for hematoxylin/eosin (HE) staining and immunohistochemistry. And **Instructive notions with respect to caring for laboratory animals** (which is released by the Ministry of Science and Technology of the People's Republic of China in September 30th, 2006.) were followed for the welfare of the animals. The animal experiments were authorized by the Animal Ethics Committee of the Experimental Animal Center of the Chongqing Medical University, Chongqing, China.

Immunohistochemical staining

The tumour tissues from nude mice, CRC and the corresponding adjacent human tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The tissue samples were sectioned into 4 μm slices, and dewaxed in a 60 °Cincubatorfor 2 h. Then, the sections were rinsedin xylene I (10 min), xylene II (10 min), xylene III (10 min), xylene IV (10 min) in sequence, and rehydrated through graded alcohol in the following order: absolute ethyl alcohol (7 min), 95%, 80%, and 70% (ethyl alcohol (5 min each)). After that, slides were boiled in citrate buffer solution for 20–25 min for antigen retrieval followed by cooling at room temperature (approximately 2–3 h), then washed three times with PBS (5 min each). The slides were then incubated in 3%

hydrogen peroxide for 10 min to neutralize endogenous peroxidase activity, and washed three times with PBS, and then blocked with 5% FBS–PBS solution at room temperature for 15 min without washing. Then, the slides were incubated with the primary antibody at 4 °C overnight. The next day, the slides were warmed to room temperature for 1 h, then washed three times with PBS (5 min each) and incubated with a secondary antibody for 30 min at 37 °C. Then the slices were washed three times with PBS (5 min each) and incubated with horseradish peroxidase-labeled streptomycin anti-biotin antibody for 30 min at 37 °C.The slides were washed three times, followed by colour development with DAB (30 s).The slides were washed with water, and the cell nuclei were dyed using hematoxylin (5 s). The slides were washed with water (15–30 min), followed by observation using light microscopy. Images were observed under a microscope at 400× light magnification.[12]

Statistical analysis

SPSS 19.0 statistical software (SPSS Inc. Chicago, IL, USA) was performed for statistical analyses. Data were analyzed with a Student's *t*-test (independent-samples t-test) and the chi-square (also termed ×2) test as appropriate. Differences were considered statistically significant with a value of $p<0.05$.

Results

CDH11 expression is downregulated in CRC cell lines and tissues

The expression levels of *CDH11* in HCT116, HT-29, LoVo, SW480, CaCo-2 and RKO colorectal tumour cell lines and normal colorectal tissues were determined using RT-PCR. *CDH11* expression was distinctly suppressed in 5 of the 6 CRC cell lines, and *CDH11* was significantly expressed in the CaCo-2 cell line (Figure 1A). The results also showed strongly expression of *CDH11* in the 4 normal colorectal tissues (Figure 1A). In addition, the messenger RNA (mRNA) expression of *CDH11* in 16 pairs of CRC and para-carcinoma tissues was measured by quantitative RT-PCR (qRT-PCR). Obviously, the expression of *CDH11* was downregulated in tumour tissues compared with the corresponding adjacent tissue ($p<0.05$) (Figure 1D). *CDH11* was located predominantly in the cell membrane. Furthermore, protein expression of *CDH11* was examined in 21 paired CRC tissues and appropriate surgical-margin tissues (adjacent tissues) by immunohistochemistry (IHC). We observed that the *CDH11* expression level was higher in appropriate surgical-margin tissues than in carcinoma tissues in 15 of the 21 paired samples (Figure 1F). The *CDH11* expression levels and the clinicopathological characteristics of CRC patients are shown in Table 2.

The CDH11 expression in CRC was downregulated due to promoter methylation and restored by its demethylation

Previous analysis showed that *CDH11*contains a typical CpG island, and suggested that *CDH11* was a likely subject of methylation-mediated silencing.[3] To identify whether *CDH11* silencing or downreglation in CRC was due to promoter methylation, CRC cell lines LoVo and HCT-116 were treated with TSA, Aza and their combination, and then RT-PCR and MSP were performed. The results showed that its expression was restored together with increasing unmethylated alleles of the *CDH11* promoter (Figure 1B). Furthermore, we analysed the methylation statues of *CDH11* by MSP in 64CRC tissues. The results of MSP showed that *CDH11* was methylated in 75.00% (48/64) of CRC tissues (Figure 1C), and revealed that the *CDH11* methylation level was specifically high in the bulk of the CRC tissues.

Overexpression of CDH11 suppresses CRC cell proliferation and colony formation

The CCK-8 and the colony formation assays were used to determine the viability-inhibiting effect of stably expressing *CDH11* in HCT116 and LoVo CRC cell lines. Overexpression of *CDH11* in HCT116 and LoVo cell lines was ascertained by RT-PCR and western blotting (Figure 2A and B).CCK-8 assay showed that overexpression of *CDH11* in HCT116 and LoVo cell lines resulted in a significant decrease in cell proliferation at 24, 48 and 72 h compared with the controls ($p<0.05$) (Figure 2C). Furthermore, the colony formation assay showed that cell transfection with *CDH11* resulted in markedly fewer and smaller colonies, and the colony numbers were reduced by 50–55% compared with transfection with the empty vector in HCT116 and LoVo cells ($p<0.001$) (Figure 2D and F). These results indicated that *CDH11* inhibited growth and viability in colorectal cancer.

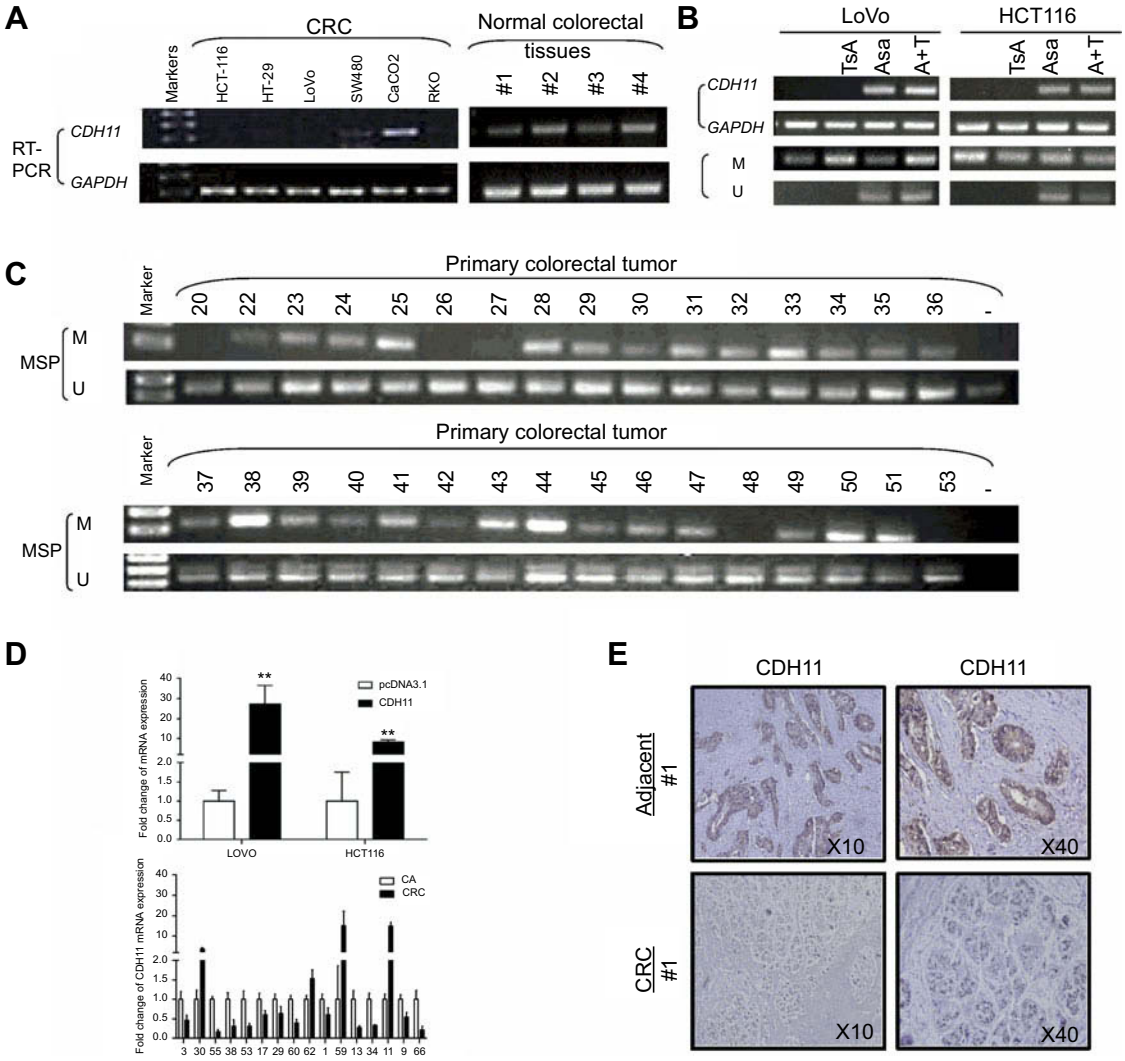

Figure 1 The expression and the methylation status of *CDH11* in CRC cell lines, primary colorectal and normal colorectal tissues. (**A**) Expression levels of *CDH11* mRNA in CRC cell lines and normal colorectal tissues and the methylation status of *CDH11* in CRC cell lines. (**B**) Restoration of *CDH11* expression by TSA, Aza and the combination CRC cell line LoVo and HCT116, and performed by RT-PCR. "M" indicates methylated *CDH11*; "U" indicates unmethylated *CDH11*. (**C**) Representative methylation of *CDH11* in primary colorectal tumour. (**D**) *CDH11* expression levels in LoVo and HCT116 cells stably expressing *CDH11* or pcDNA 3.1 and 16 pairs of CRC and adjacent tissues were measured by quantitative RT-PCR (qRT-PCR). (**E**) Representative images of *CDH11* IHC in paried CRC tissues and adjacent tissues (magnification ×200 and ×400).
Abbreviations: CA, colorectal tumor adjacent tissues; CRC, colorectal cancer tissues.

CDH11 induces cell cycle arrest in the G0/G1 phase and apoptosis in CRC cell lines

To determine how *CDH11* affects cell proliferation in CRC, the cell cycle and apoptosis analysis were performed by flow cytometry. The result shows a significantly increased number of *CDH11*-expressing cells accumulating in the G0/G1 phase of the cell cycle compared with the control cells ($p<0.01$, $p<0.001$) (Figure 3A), indicating that the inhibition of cell proliferation by *CDH11* was likely mediated by a cell cycle delay at theG0/G1 phase. Subsequently, the rate of apoptosis was detected using Annexin V-FITC/PI staining assay. The flow cytometry results confirmed that the number of Annexin V-PI-positive cells markedly increased in *CDH11*-transfected cells compared with controls ($p<0.001$ and $p<0.05$) (Figure 3B). Overall, these results suggested that the inhibition of cell proliferation by *CDH11* is due to its mediation of the cell cycle arrest at the G0/G1 phase and induction of apoptosis.

CDH11 inhibits cell migration and invasion in colorectal tumour cells

Wound-healing and transwell assays were performed to further investigate the effects of *CDH11* on cell migration

Table 2 The clinical information of colorectal carcinoma tissues

No.	Age	Size (cm)	Gender (1M2F)	Grade (1234X)	T-stage	N-status	lymph metastasis	Stage	Distant metastasis	Localisation	Athological type
C1	64	3	1	x	3	N1	3	IIIB	3	0	Ad
C3	78	2	2	2	3	N1	1	IIIB	0	0	Ad
C9	59	3	1	2	3	N0	0	IIA	1	0	Ad
C11	60	2	2	2	3	N0	0	IIA	0	0	Ad
C13	72	3	2	2~3	3	N0	0	IIA	0	0	Ad
C17	79	2	2	2	3	N2	4	IIIB	4	0	Ad
C29	65	2	2	2	3	N2	5	IIIB	5	0	Ad
C30	78	3	1	2	3	N1	1	IIIB	1	0	Ad
C34	70	2	1	2~3	2	N0	0	I	0	0	Ad
C38	76	2	2	2	2 3	N1	1	IIIB	1	0	Ad
C53	80	5,6	1	2	4	N0	0	IV	0	1	Ad
C55	70	6	1	2	3	N0	0	IV	0	1	Ad
C59	66	3,2	1	2	3	N1	2	IV	2	1	AD
C60	74	10,6	2	2	3	N1	1	IV	1	1	MA
C62	74	3,4	2	2	3	N1	2	III	2	0	Ad
C66	79	4,3	1	1~2	2	N0	0	I	0	0	Ad

Abbreviations: MA, mucinous adenocarcinoma; Ad, adenocarcinoma.

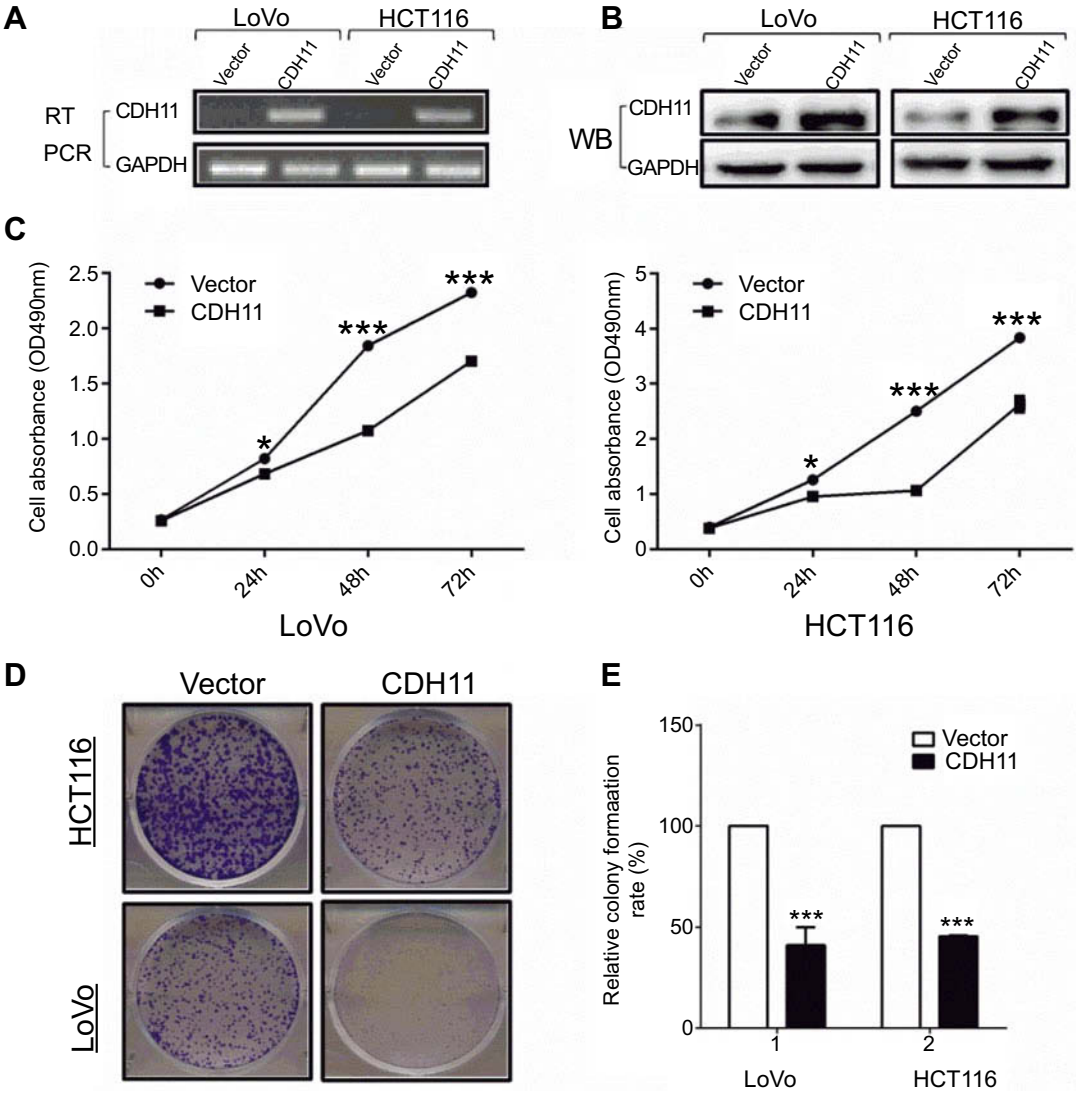

Figure 2 Overexpression of CDH11 inhibits the cell proliferation and colony formation in LoVo andHCT116 cell lines. (**A**) Expression of CDH11 in vector- and CDH11-transfected cells by RT-PCR. (**B**) Expression of CDH11by western blot analysis in vector- and CDH11-transfected in CRC cells. (**C**) CCK-8 assay for cell proliferation of vector-and CDH11-infected CRC cell lines. ***p<0.001, *p<0.05. (**D**) Representative colony formation assay in LoVo and HCT116 cells infected with the control vector and CDH11-expressing vector. (**E**) Quantitative analysis of colony formation (***p<0.001).

and invasion in CRC. The results of wound-healing assay showed that, compared with the vector-transfected cells, *CDH11* stablye-expressing cells migrated along the wound borderline significantly slower, as observed for cells at LoVo 0 h, 24 h, 48 h and 72 h (p<0.001) and HCT116 cells at 0 h, 24 h,48 h and 60 h (p<0.05) (Figure 4A and B). The transwell assay both with and without a Matrigel barrier further illustrated that the numbers of migratedor invaded *CDH11*-overexpressing in LoVo and HCT116 cells were significantly reduced compared with control groups(p<0.001) (Figure 4C and D). These results determined that *CDH11* inhibits cell migration and invasion in colorectal tumour cells.

CDH11 suppresses the growth of tumuor xenografts in nude mice

To further identify if *CDH11* inhibits the CRC growth, in vivo tumourigenicity test were carried out. Stably-transfected and vector-transfected HCT116 were injected to form tumours in nude mice (Figure 5A). Tumours were removed from nude mice 19 days after injection (Figure 5B), the measurement results showed that the mean sizes and weights of tumours formed *CDH11* stable cells were markedly lower compared with the control groups (p<0.001, p<0.05) (Figure 5C and D). Hematosylin & eosin (H&E) staining and immunohistochemistry (IHC)

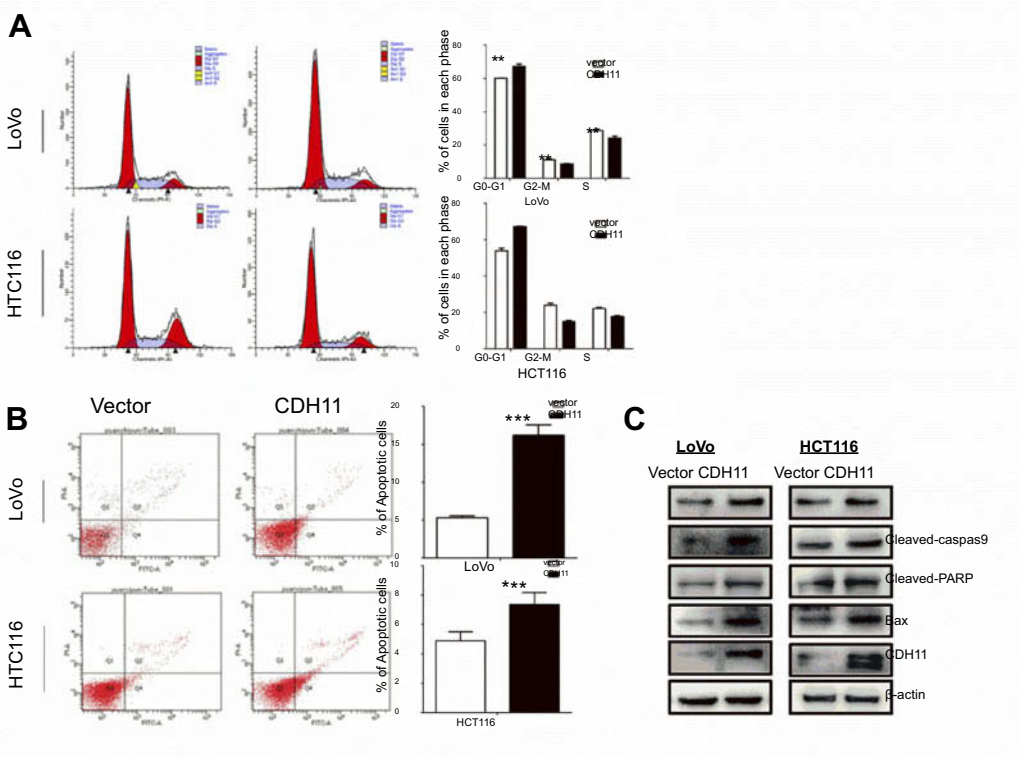

Figure 3 Flow cytometric analysis. (**A**) Overexpression of CDH11induced LoVo and HCT116 cell cycle arrest at G0-G1. Left: Representative distribution of cell cycles. Right: statistical summary of flow cytometry data. Values were assessed by Student's t-test. The graph displays the mean ± SD of three independent experiments (**p<0.01); (***p<0.001). (**B**) The apoptosis rate of control vector andectopic expression of CDH11 in LoVo and HCT116 cell was detected by flow cytometric analysis with Annexin V-FITC and PI-staining (***p<0.001). (**D**) Western blot analysis of four classic markers of apoptosis, cleaved-PARP, Bax, cleaved-caspase 3, cleaved-caspase 9 in vector- and CDH11-transfectedLoVo and HCT116 cells, **β**-actin as a control.

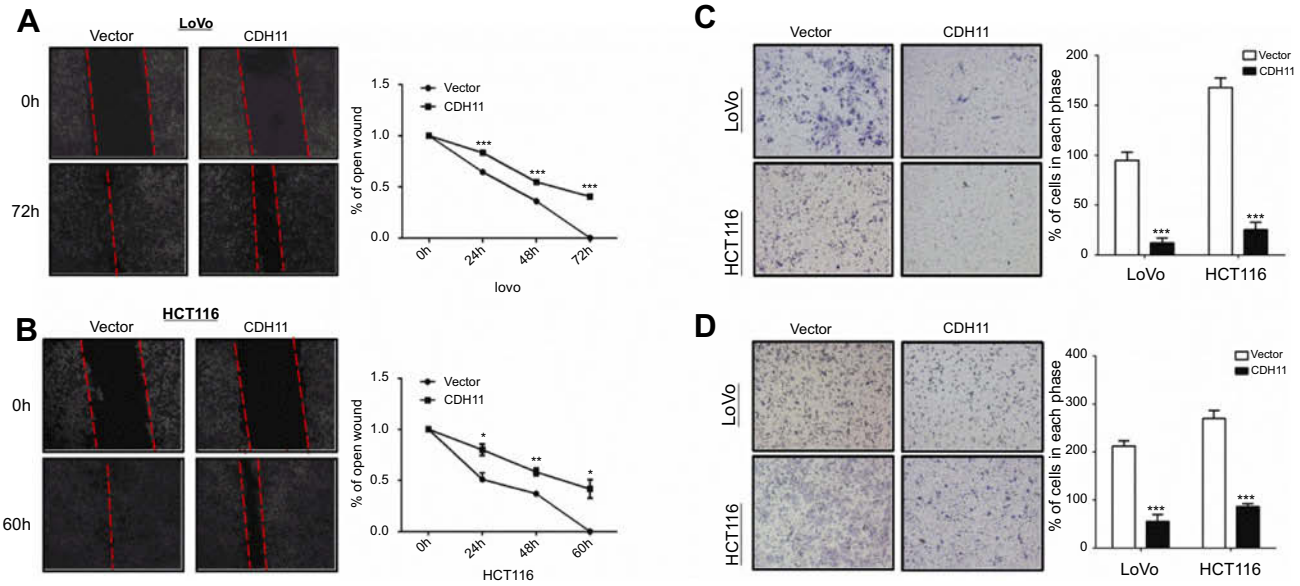

Figure 4 Ectopic expression of CDH11 inhibited the cell motility in LoVo and HCT116 cells. (**A** and **B**) Representative images of wound healing assay were captured at 0, 48 or 72 h, and quantitative analysis of migration length of vector- and CDH11-transfered LoVo and HCT116 cells, shown as means ± SD. ***p<0.001, **p<0.01, *p<0.05. And the suppressive effects of CDH11 on cell migration and invasion in CRC cells were investigated by transwell assays. Representative images of transwell (**C**) invation and (**D**) migration assays (magnification ×400) and quantitative analysis of the number of migrating and invasion cells (***p<0.001).

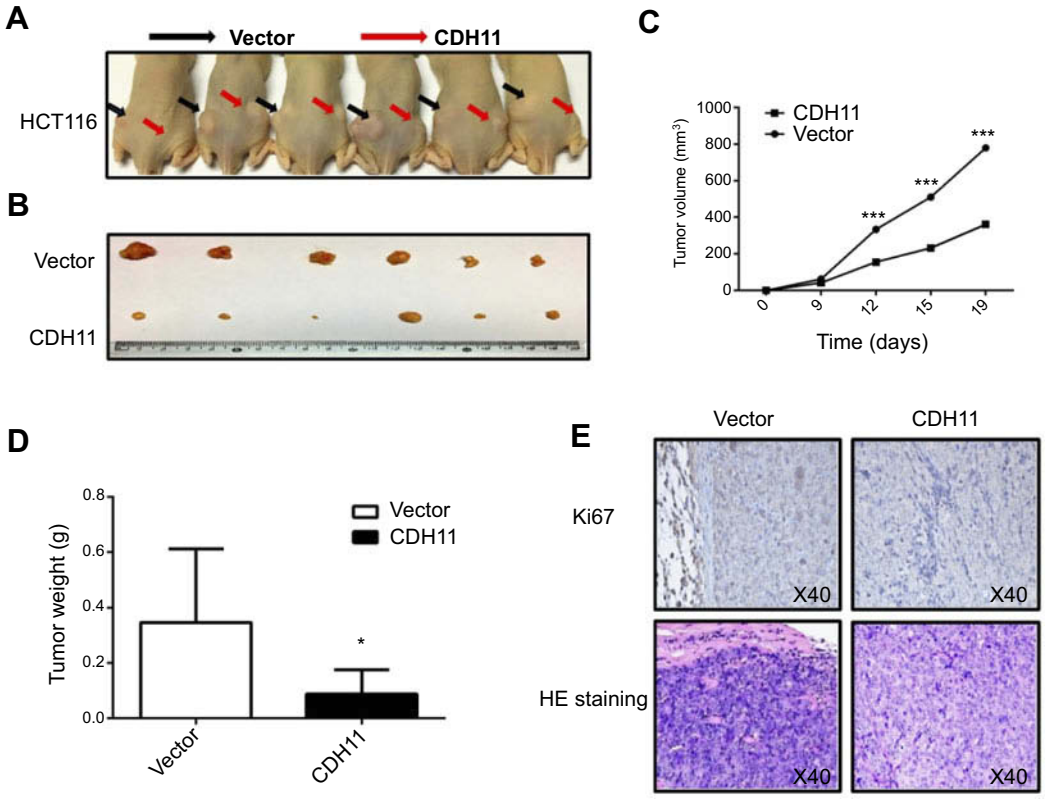

Figure 5 *CDH11* suppresses the growth of tumor xenografts in nude mice. (**A** and **B**) Images of tumor xenografts in nude mice. (**C**) Tumor growth curve for vector-and *CDH11*-infected tumors in nude mice (*p<0.05). (**D**) Tumor weight of *CDH11*-infected cells in nude mice compared with control tumors (*p<0.05). (**E**) Representative images of IHC analysis of the expression of *Ki67*and HE staining in tumours from nude mice (magnification ×400).

were performed to analyse the tumour features and expression of *CDH11* protein and proliferation-associated protein Ki-67 (Figure 5E). *Ki-67* expression was significantly downregulatied in tumours overexpressing *CDH11*compared with control. These data confirmed that *CDH11*acts as a TSG in CRC tumourigenesis in vivo.

CDH11 antagonized the NF-kB signaling pathways

A previous study has shown that *CDH11* functions as a pro-apoptotic tumour suppressor by antagonizing the *Wnt/β*-catenin and *AKT/Rho A* signaling pathway.[3] Several reports have suggested that *NF-kB* signaling is frequently affected in CRC.[17–20] And *Bcl-2* and *Bcl-XL* are downstream target genes of the *NF-kB* pathway. Here, we investigated whether *CDH11* functions as a tumour suppressor through the *NF-kB* signaling pathway. Our results showed that phospho-*NF-kB* were markedly downregulated by overexpressing *CDH11*. Furthermore, overexpression *CDH11* resulted in an obvious reduction of *Bcl-2* and *Bcl-XL*. All of those indicated that *CDH11* suppressed the CRC carcinogenic process by inactiving *NF-kB* signaling pathway (Figure 6).

Discussion

Cadherin-11 expression has been studied in many solid tumors. But *CDH11* in CRC has not been still well elucidated. Increasing evidence has revealed that *CDH11* plays a momentous role in the development of a broad series of human malignancies, and identified *CDH11* as a functional tumour suppressor which is commonly silenced by promoter methylation.[3] Previous studies have shown that *CDH11* inhibits invasion and proliferation in head and neck cancer,[21] osteosarcoma,-[8,22] glioma,[23] melanoma[24] and bladder cancer.[25] The correlation between the function of *CDH11* and related mechanisms in colorectal cancer remained unclear. In this study, we discovered that *CDH11* was expressed in normal colon tissues, but frequently silenced or downregulated in CRC cell lines. These above results suggested that the expression of *CDH11* is downregulated in CRC.

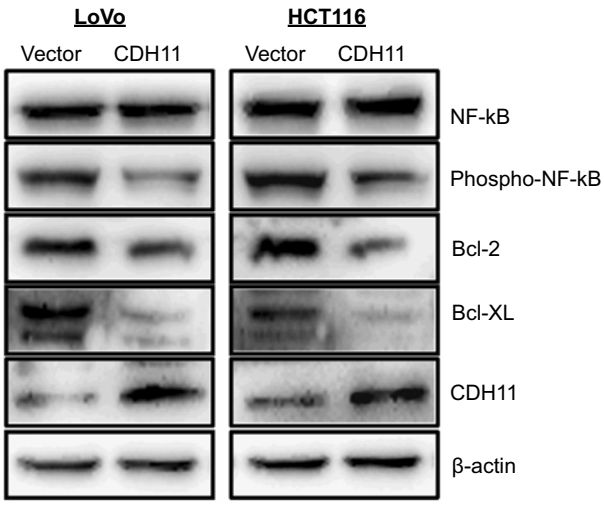

Figure 6 Overexpression of CDH11 antagonized NF-kB signaling pathway. Western blot was performed using antibodies against NF-kB, phospho-NF-kB and its downstream targets Bcl-2 and Bcl-XL; β-actin was used as a control.

It is known that promoter methylation is a primary mechanism involved in gene changes such as gene absent or gene downregulation, which has been found in multiple human tumours. The results of this study suggested that the expression of *CDH11* in CRC is epigenetically inactivated by promoter-specific methylation in CRC. The results of MSP revealed that the *CDH11* methylation level was specifically high in the bulk of the CRC tissues. And the demethylation treatment A + T assay showed that its expression was restored together with increasing unmethylated alleles of the *CDH11* promoter.

Previous studies reported that *CDH11* inhibited tumour cell clonogenicity and induced apoptosis.[3] We investigated the biological functions of *CDH11* by both in vivo and in vitro assays. The results of the present study ascertain that overexpression of *CDH11* induces cell cycle arrest in the G0/G1 phase and apoptosis and suppresses CRC cell proliferation and colony formation in both HCT116 and LoVo CRC cell lines. In addition, it has been reported that ectopic-expression of *CDH11* inhibits invasion in head and neck cancer.[21] Consistent with these previous reports, we found that ectopic-expression of *CDH11* inhibited migration and invasion in colorectal tumour cells. Subsequently, an in vivo tumourigenicity test in nude mice was carried out to confirm the inhibitory effect of *CDH11* on CRC growth. In short, all these results together strongly suggest that *CDH11* functions as a tumour suppressor in CRC cells.

Previous study found that *CDH11* functions as a TSG through antagonizing *Wnt/β*-catenin and *AKT/Rho A* signaling.[3] And some early studies showed that the re-expression of *CDH1*, one member of cadherin superfamily, may depend on *NF-kB* activation, and *NF-kB* promotes migration and invasion by re-expression of *CDH1* in cholangiocarcinoma cells.[26] Whether *CDH11*, the member of cadherin superfamily, performs by a similar mechanism that acts on the *NF-kB* pathway in CRC remains unclear. In this study, we investigated whether *CDH11* functions as a tumor suppressor in CRC through the *NF-kB* signaling pathway.

Nuclear factor kappa B (*NF-kB*), as a pleotropic transcription factor, regulates expression of a number of genes that promote multiple cancer cells growth, survival and neoplastic transformation.[27–30] In this signaling pathway, transcriptionally competent *NF-kB* is a heterodimer composed of *p65* (*NF-kB*) and p50 subunits,[31,32] and this heterodimer is insulated in the cytoplasm by *p65*(*NF-kB*) bound IkBα[27,33] in unstimulated cells. Phospho-*NF-kB* is a key component in the *NF-kB* pathway, where it suppresses cell survival and growth, and *Bcl-2* and *Bcl-XL* are downstream target genes of the *NF-kB* pathway.[34,35] Our results showed that when compared to the empty vector group, the expression levels of phospho-*NF-kB* were markedly downregulated in the *CDH11* group, and *CDH11* resulted in an obvious reduction of *Bcl-2* and *Bcl-XL*, indicating that *CDH11* may affect the CRC carcinogenic process by inactiving *NF-kB* signaling pathway. Further rescue t trials are needed to identify the role of *NF-kB* signaling pathway in the *CDH11* acting mechanism.

However, the target genes of *CDH11* were not identified in this study, which may therefore assist in identifying its role in the progression of CRC. And the number of

clinical samples of CRC used in this study was insufficient, so we could not determine the possible relationship between *CDH11* methylation and the pathological features and survival prognoses of CRC. Further studies of more clinical samples are warranted to explore the mechanism of *CDH11* and to confirm that *CDH11* is a prognostic marker for CRC.

Conclusion

In summary, our study suggests that *CDH11* downregulation in CRC results from promoter methylation. And *CDH11* induces cell cycle arrest in the G0/G1 phase and apoptosis to suppressing CRC cell proliferation and colony formation, thus suppressing tumour cell proliferation, migration and invasion, probably by affecting the *NF-kB* signaling pathway. Overall, *CDH11* may be considered as a functional TSG in CRC, and has the potential to serve as a valuable prognostic marker for colorectal cancer.

Abbreviation list

CDH11, cadherin-11; CRC, colorectal cancer; qRT-PCR, quantitative RT-PCR; MSP, methylation-specific PCR; TSG, tumor suppressor gene; aCGH, array comparative genomic hybridization; mRNA, messenger RNA; IHC, Immunohistochemistry; H&E, Hematosylin & eosin; NFkB, Nuclear factor kappa B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DNMT, DNA methyltransferase; Aza, 5-Aza-2′-deoxycytidine; TSA, trichostatin A; PBS, phosphate-buffered saline; PI, propidium iodide; WB, Western blot.

Acknowledgments

The authors thank Professor Qian Tao (the Chinese University of Hong Kong, Hong Kong, China) for generously providing CRC cell lines, plasmid and primers, and thank Professor Tingxiu Xiang technical assistance, and help with experimental design. This research was funded by National Natural Science Foundation of China, grant number 3142010391.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;394–424.
2. Rabeneck L, Horton S, Zauber AG, Earle C. Colorectal cancer. In: *Cancer. Disease Control Priorities*. 3rd ed. The International Bank for Reconstruction and Development / The World Bank Vol. 363. 2015:101–116.
3. Li L, Ying J, Li H, et al. The human cadherin 11 is a pro-apoptotic tumor suppressor modulating cell stemness through Wnt/beta-catenin signaling and silenced in common carcinomas. *J Cell Sci*. 2012;31(34):3901–3912.
4. Margulis A, Zhang W, Alt-Holland A, Crawford HC, Fusenig NE, Garlick JA. E-cadherin suppression accelerates squamous cell carcinoma progression in three-dimensional, human tissue constructs. *Cancer Res*. 2005;65(5):1783–1791. doi:10.1158/0008-5472.CAN-04-3399
5. Andreeva AV, Kutuzov MA. Cadherin 13 in cancer. *Genes Chromosomes Cancer*. 2010;49(9):775–790.
6. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology*. 2008;135(4):1079–1099. doi:10.1053/j.gastro.2008.07.076
7. Assefnia S, Dakshanamurthy S, Guidry Auvil JM, et al. Cadherin-11 in poor prognosis malignancies and rheumatoid arthritis: common target, common therapies. *Oncotarget*. 2014;5(6):1458–1474. doi:10.18632/oncotarget.1538
8. Deng Z, Niu G, Cai L, Wei R, Zhao X. The prognostic significance of CD44V6, CDH11, and beta-catenin expression in patients with osteosarcoma. *Biomed Res Int*. 2013;2013:496193. doi:10.1155/2013/496193
9. Yao J, Deng B, Zheng L, Dou L, Guo Y, Guo K. miR-27b is upregulated in cervical carcinogenesis and promotes cell growth and invasion by regulating CDH11 and epithelial-mesenchymal transition. *Oncol Rep*. 2016;35(3):1645–1651. doi:10.3892/or.2015.4500
10. Carmona FJ, Villanueva A, Vidal A, et al. Epigenetic disruption of cadherin-11 in human cancer metastasis. *J Pathol*. 2012;228(2):230–240. doi:10.1002/path.4011
11. Wang C, Yue Y, Shao B, et al. Dickkopf-related protein 2 is epigenetically inactivated and suppresses colorectal cancer growth and tumor metastasis by antagonizing Wnt/β-catenin signaling. *Cell Physiol Biochem*. 2017;41(5):1709–1724. doi:10.1159/000471861
12. Xiang S, Xiang T, Xiao Q, Li Y, Shao B, Luo T. Zinc-finger protein 545 is inactivated due to promoter methylation and functions as a tumor suppressor through the Wnt/beta-catenin, PI3K/AKT and MAPK/ERK signaling pathways in colorectal cancer. *Int J Oncol*. 2017;51(3):801–811. doi:10.3892/ijo.2017.4064
13. Tao Q, Huang H, Geiman TM, et al. Defective de novo methylation of viral and cellular DNA sequences in ICF syndrome cells. *Hum Mol Genet*. 2002;11(18):2091–2102.
14. Pei L, He X, Li S, et al. KRAB zinc-finger protein 382 regulates epithelial-mesenchymal transition and functions as a tumor suppressor, but is silenced by CpG methylation in gastric cancer. *Int J Oncol*. 2018. doi:10.3892/ijo.2018.4446
15. Zhao L, Li S, Gan L, et al. Paired box 5 is a frequently methylated lung cancer tumour suppressor gene interfering beta-catenin signalling and GADD45G expression. *J Cell Mol Med*. 2016;20(5):842–854. doi:10.1111/jcmm.12768
16. Li C, Tang L, Zhao L, et al. OPCML is frequently methylated in human colorectal cancer and its restored expression reverses EMT via downregulation of smad signaling. *Am J Cancer Res*. 2015;5(5):1635–1648.
17. Elshaer M, Chen Y, Wang XJ, Tang X. Resveratrol: an overview of its anti-cancer mechanisms. *Life Sci*. 2018;207:340–349. doi:10.1016/j.lfs.2018.06.028
18. Hai Ping P, Feng BT, Li L, Nan Hui Y, Hong Z. IL-1beta/NF-kb signaling promotes colorectal cancer cell growth through miR-181a/PTEN axis. *Arch Biochem Biophys*. 2016;604:20–26. doi:10.1016/j.abb.2016.06.001
19. Puvvada SD, Funkhouser WK, Greene K, et al. NF-kB and Bcl-3 activation are prognostic in metastatic colorectal cancer. *Oncology*. 2010;78(3–4):181–188. doi:10.1159/000313697

20. De Simone V, Franze E, Ronchetti G, et al. Th17-type cytokines, IL-6 and TNF-alpha synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. *Oncogene*. 2015;34(27):3493–3503. doi:10.1038/onc.2014.286
21. Piao S, Inglehart RC, Scanlon CS, Russo N, Banerjee R, D'Silva NJ. CDH11 inhibits proliferation and invasion in head and neck cancer. *J Oral Pathol Med*. 2017;46(2):89–97. doi:10.1111/jop.12471
22. Azarsina S, Otoukesh B, Taheriazam A, et al. Diagnostic investigations of PLA2G16 and CDH11 expression levels as independent prognostic markers of human osteosarcoma. *Arch Med Sci*. 2017;13(6):1347–1351. doi:10.5114/aoms.2016.59710
23. Delic S, Lottmann N, Jetschke K, Reifenberger G, Riemenschneider MJ. Identification and functional validation of CDH11, PCSK6 and SH3GL3 as novel glioma invasion-associated candidate genes. *Neuropathol Appl Neurobiol*. 2012;38(2):201–212. doi:10.1111/j.1365-2990.2011.01207.x
24. Bosserhoff AK, Ellmann L, Quast AS, Eberle J, Boyle GM, Kuphal S. Loss of T-cadherin (CDH-13) regulates AKT signaling and desensitizes cells to apoptosis in melanoma. *Mol Carcinog*. 2014;53(8):635–647. doi:10.1002/mc.22018
25. Lin YL, Gui SL, Ma JG. Aberrant methylation of CDH11 predicts a poor outcome for patients with bladder cancer. *Oncol Lett*. 2015;10(2):647–652. doi:10.3892/ol.2015.3337
26. Zhang K, Zhaos J, Liu X, et al. Activation of NF-B upregulates Snail and consequent repression of E-cadherin in cholangiocarcinoma cell invasion. *Hepato-gastroenterology*. 2011;58(105):1–7.
27. Jana A, Krett NL, Guzman G, et al. NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. *Oncotarget*. 2017;8(23):37377–37393. doi:10.18632/oncotarget.16343
28. Wang S, Liu Z, Wang L, Zhang X. NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol Immunol*. 2009;6(5):327–334. doi:10.1038/cmi.2009.43
29. Voboril R, Weberova-Voborilova J. Constitutive NF-kappaB activity in colorectal cancer cells: impact on radiation-induced NF-kappaB activity, radiosensitivity, and apoptosis. *Neoplasma*. 2006;53(6):518–523.
30. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*. 1996;14:649–683. doi:10.1146/annurev.immunol.14.1.649
31. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. 2002;109(Suppl):S81–96.
32. Hassanzadeh P. Colorectal cancer and NF-kappaB signaling pathway. *Gastroenterol Hepatol Bed Bench*. 2011;4(3):127–132.
33. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev*. 2006;210:171–186. doi:10.1111/j.0105-2896.2006.00375.x
34. Deeb D, Gao X, Liu YB, Pindolia K, Gautam SC. Pristimerin, a quinonemethide triterpenoid, induces apoptosis in pancreatic cancer cells through the inhibition of pro-survival Akt/NF-κB/mTOR signaling proteins and anti-apoptotic Bcl-2. *Int J Oncol*. 2014;44(5):1707–1715. doi:10.3892/ijo.2014.2325
35. Ghaderian SB, Hayati F, Shayanpour S, Beladi Mousavi SS. Diabetes and end-stage renal disease; a review article on new concepts. *J Renal Inj Prev*. 2015;4(2):28–33. doi:10.12861/jrip.2015.07